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Ultrastructural Analysis of Spermiogenesis and Sperm Morphology in *Chorus giganteus* (Lesson, 1829) (Prosobranchia: Muricidae)

by

ROBERTO JARAMILLO, ORLANDO GARRIDO, AND BORIS JORQUERA

Instituto de Embriología, Facultad de Ciencias, Universidad Austral de Chile, Casilla 567, Valdivia, Chile

Abstract. Spermatid differentiation and the morphology of mature spermatozoa in the gastropod *Chorus giganteus* (Lesson, 1829) were investigated. Five phases of spermiogenesis are proposed based on the polarization of organelles, nuclear elongation, and chromatin condensation. The ultrastructure of the spermatozoa of *Chorus giganteus* is compared to those of other Muricidae. Possible phylogenetic and functional relationships among prosobranch spermatozoa are discussed.

INTRODUCTION

STUDIES DEALING with spermatogenesis in mollusks have shown close relationships between sperm morphology and certain aspects of reproductive strategy. In particular, sperm dimorphism appears correlated with the presence of nutritive eggs in prosobranchs (PORTMAN, 1931a; TUZET, 1930; NISHIWAKI, 1964; TOCHIMOTO, 1967). In the species studied, both normal (or typical) spermatozoa and abnormal (or atypical) spermatozoa have been recognized. The latter can be oligopyrene (*i.e.,* with a small quantity of chromatin) or apyrene (*i.e.,* with no chromatin) (PLATNER, 1889; AUERBACH, 1896; MEVES, 1903). These atypical spermatozoa could play a role in the feeding of normal embryos by giving rise to abortive embryos. However, all prosobranchs that utilize nutritive eggs do not exhibit sperm dimorphism, nor do all those prosobranchs that exhibit sperm dimorphism utilize nutritive eggs (O. HYMAN, 1925; L. HYMAN, 1967; ANKEL, 1930; PORTMAN, 1927, 1931b).

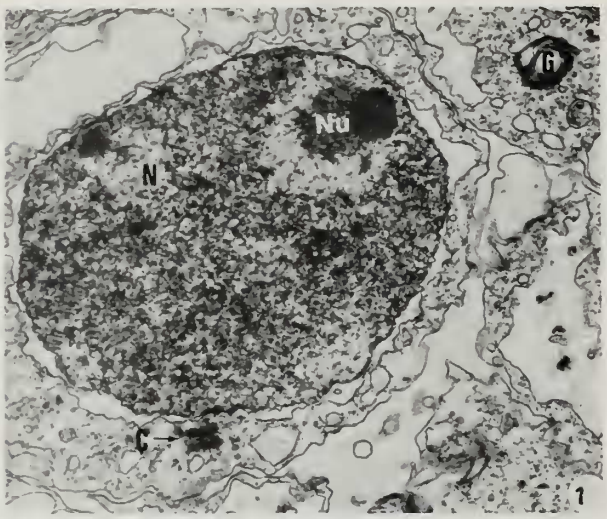
A second correlation has been established between the morphology of typical spermatozoa and the nature of the medium in which fertilization occurs (TUZET, 1950; FRANZÉN, 1955, 1956, 1970; FAWCETT, 1970). FRANZÉN (1955), after studying 15 species of gastropods, has classified spermatozoa into two types. Type I, or primitive spermatozoa, belong to species with external fertilization; these have a cone-shaped head and a short middle piece containing mitochondria at the base of the nucleus. Type II, or modified spermatozoa, are found in species with internal fertilization; they display a threadlike head, an elongate middle piece, and mitochondria arranged around the axial filament. NISHIWAKI (1964) reported that some Japanese prosobranchs also have typical and atypical spermatozoa, which he classified as typical spermatozoa of types I and II in keeping with Franzén's original scheme. Members of the Neogastropoda have internal fertilization (HYMAN, 1967; FRETTER & GRAHAM, 1962) and most neogastropod spermatozoa are considered as a modified type.

Ultrastructural analyses of spermiogenesis and mature spermatozoa have been reported for the muricid prosobranchs *Nucella lapillus* (WALKER & MACGREGOR, 1968; WALKER, 1970) and *Concholepas concholepas* (HUAQUÍN & BUSTOS-OBREGÓN, 1981) and the buccinid prosobranch *Colus stimpsoni* (WEST, 1978). In histological studies of *Nucella lapillus* (PORTMAN, 1931a) and *Chorus giganteus* (AMÍN *et al.,* 1984), some aspects of spermatogenesis were included.

However, there are no ultrastructural analyses of spermiogenesis and mature spermatozoa in *Chorus.* This paper identifies the type of spermatozoa in *Chorus giganteus* and correlates the type with functional and phylogenetic aspects of the animal's reproductive strategy.

MATERIALS AND METHODS

Mature specimens of *Chorus giganteus* were collected by diving at Puerto Claro, Valdivia (39°53'S, 73°22'W) in different seasons of the year.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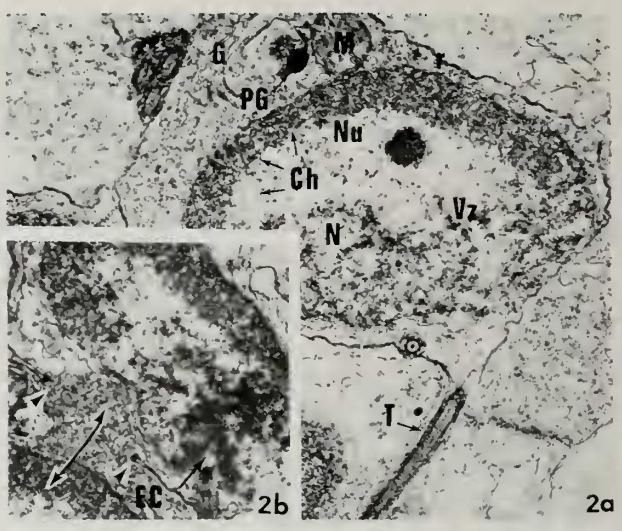
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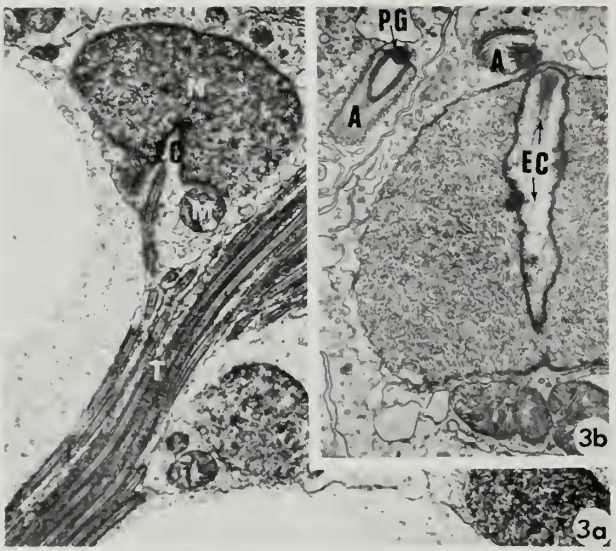
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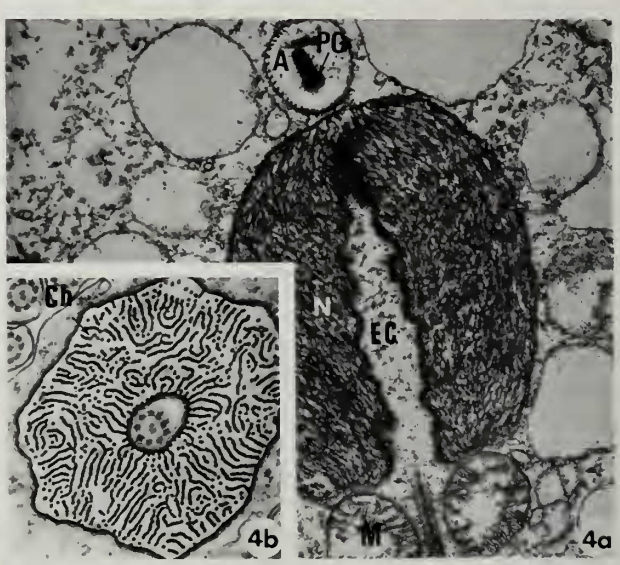
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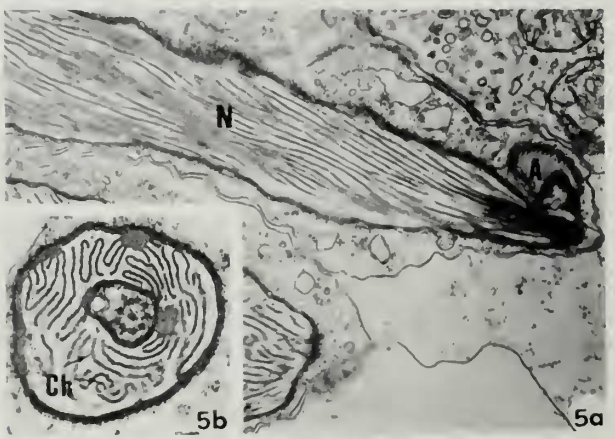
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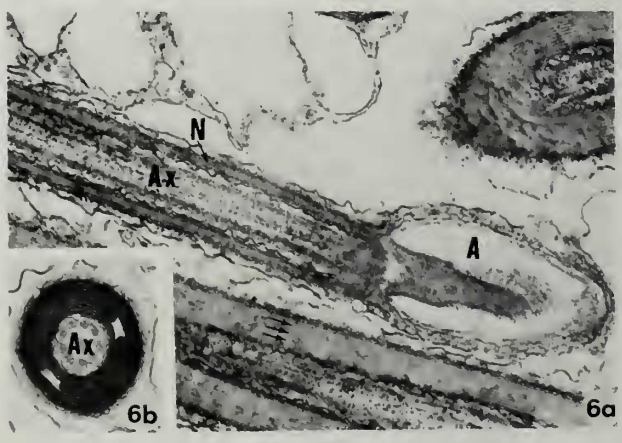
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Pieces of testis and seminal vesicles (Amín *et al.*, 1984) were fixed for 2 h in a three-fold aldehyde mixture containing 2.5% glutaraldehyde, 10% para-formaldehyde, and 2% acrolein, buffered to pH 7.2 with 0.2 M phosphate (Rodríguez, 1969). After washing with the same phosphate buffer, tissues were postfixed for 2 h in buffered 1% OsO_4 and embedded in an epon-araldite mixture (Richardson *et al.*, 1960). Ultrathin sections were stained either (1) with uranyl acetate and lead citrate (Glauert, 1965) or (2) according to the method of Thiery & Rambourg (1974) for the demonstration of polysaccharides. In the last method, sections were treated with 1% periodic acid solution, rinsed in three successive changes of distilled water, and refloated on the surface of 1% thiosemicarbazide in 10% acetic acid; sections were then rinsed thoroughly in distilled water, refloated in 1% aqueous silver proteinate solution, rinsed in distilled water, and mounted on copper grids. Smears of mature spermatozoa, obtained by puncturing the seminal vesicles, were fixed as described above and prepared for light microscopy (LM) and scanning electron microscopy (SEM). For LM, smears were stained with hematoxylin-eosin and, for SEM, they were dehydrated in acetone, critical point dried, and coated with gold.

Observations were done with a Philips 300 (TEM) and Hitachi H-700 (TEM and SEM).

RESULTS

Ultrastructural Changes during Spermiogenesis

Spermiogenesis in sections of *Chorus giganteus* testis was studied ultrastructurally, and the process divided into 5 phases.

Phase 1 involves polarization of the centriole. Initial spermatids were small, rounded cells, 8 μm in diameter, grouped into clusters of approximately 8 cells linked by cytoplasmic bridges. Each had a spherical nucleus, 3 to 4 μm in diameter; chromatin was homogeneously distributed and a vacuolization zone surrounded an excentric nucleolus. A typical Golgi apparatus was formed by 7–8 curved saccules facing the nucleus. Small vesicles associated with the tips of the saccules accumulated on the concave side of the complex. A single centriole occupied a position opposite to that of the nucleolus, thus establishing the cell's polar axis. Adjacent to the centriole the nuclear membrane presented a thickening of nuclear material. Some mitochondria also were seen (Figure 1).

Phase 2 is the polarization of the Golgi apparatus. During this phase the nucleus became ovoid, with the major axis perpendicular to the cell's polar axis. A semilunar-shaped zone of condensed chromatin appeared in the apical nuclear pole and the vacuolization zone around the nucleolus became larger than in phase 1 (Figure 2a).

The Golgi complex migrated to the apical cytoplasm, near the position of the nucleolus. During this migration the small vesicles associated with the concave side of the Golgi saccules produced an electron-dense proacrosomal granule (Figure 2a). The remaining cytoplasm contained scattered mitochondria. An invagination at the nuclear basal pole progressively elongated and became the endonuclear channel. This channel was delimited by a thickening on the inner side of the nuclear membrane. The centriole was located inside the channel and gave rise to an axoneme of 9+2 microtubules, which upon elongation forms the tail. Individual spermatids still remained joined by cytoplasmic bridges (Figure 2b).

Phase 3 is the polarization of mitochondria. In the cytoplasm, mitochondria were grouped in the vicinity of the nuclear base forming a single mitochondrial annulus corresponding to the developing middle piece. The tail continued its elongation and endonuclear channel formation ended (Figures 3a, b).

Condensed chromatin was homogeneously distributed throughout the nucleus, and a nucleolus was no longer seen. Over the proacrosomal granule, the Golgi apparatus formed the early double membrane acrosomic vesicle. At the inner surface of the outer acrosomal membrane appeared a dense crest, giving the membrane a helicoidal appearance (Figure 3b). The major axis of the vesicle was perpendicular to the cell's polar axis.

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Explanation of Figures 1 to 6

Figure 1. Spermatid, phase 1. N, nucleus; Nu, nucleolus; G, Golgi apparatus; C, centriole. ×16,000.

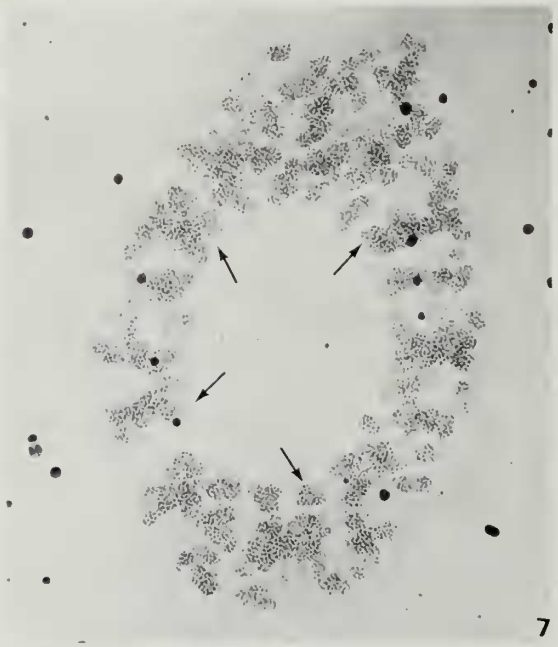
Figure 2. Spermatid, phase 2. 2a. N, nucleus; Nu, nucleolus; G, Golgi apparatus; PG, proacrosomal granule; M, mitochondria; T, tail; Ch, chromatin; Vz, vacuolization zone. ×12,000. 2b. Arrows show a cytoplasmic bridge; EC, endonuclear channel. ×18,000.

Figure 3. Spermatid, phase 3. 3a. N, nucleus; M, mitochondria; EC, endonuclear channel; T, tail. ×10,000. 3b. A, acrosome; PG, proacrosomal granule; EC, endonuclear channel. ×12,000.

Figure 4. Spermatid, phase 4. 4a. N, nucleus; A, acrosome; PG, proacrosomal granule; M, mitochondria; EC, endonuclear channel. ×16,000. 4b. Cross section of spermatid. Ch, chromatin radially arranged. ×18,000.

Figure 5. Spermatid, in early phase 5. 5a. A, acrosome; N, nucleus. ×18,000. 5b. Cross section of the head of a spermatid with initial rearrangement of chromatin (ch). ×34,000.

Figure 6. Spermatid, in late phase 5. 6a. A, acrosome; Ax, axoneme; N, nucleus; arrows show the lamellar distribution of the chromatin around the nucleus. ×29,000. 6b. Cross section of the head of a spermatid with concentric lamellae of chromatin. Ax, axoneme. ×30,000.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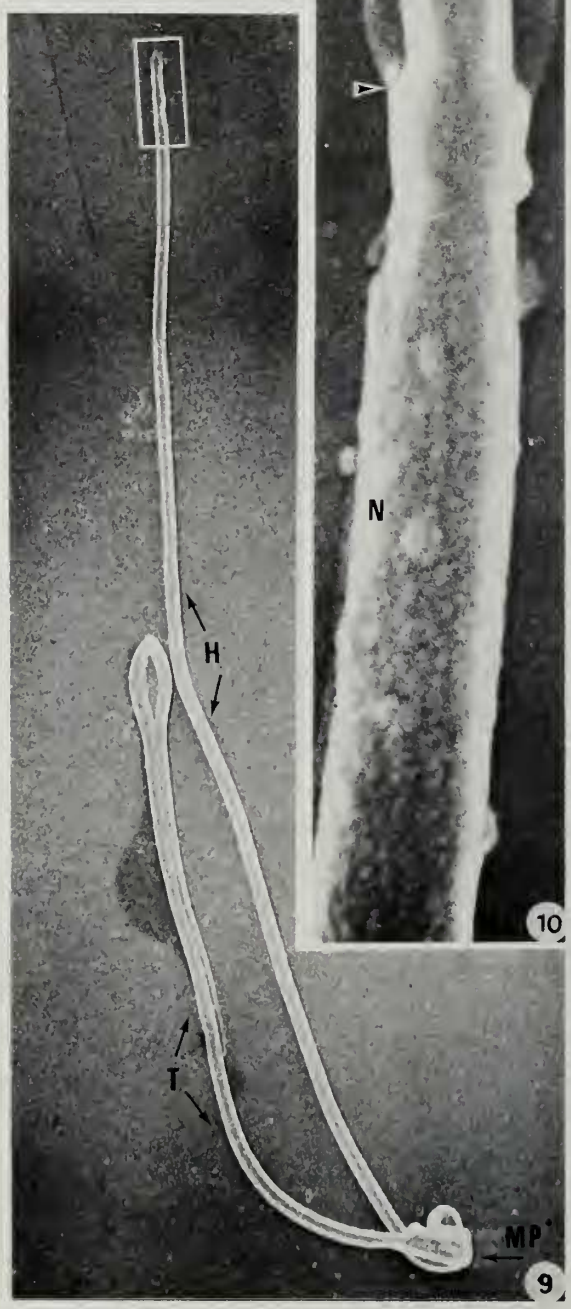
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Phase 4 is nuclear elongation. The nucleus elongated along the cell's polar axis to establish a 2:1 length-diameter ratio (Figure 4a). Chromatin appeared, forming irregularly arranged longitudinal filaments that later fused in a lamellar fashion radial to the endonuclear channel (Figure 4b). The middle piece showed no modifications, but tail elongation continued. Between the cells cytoplasmic bridges were not observed.

Phase 5 is termed the lamellar chromatin phase. During this time the nucleus, now approximately 0.6 μm in diameter in its middle region, continued its elongation. The chromatin lamellae lost their radial appearance and became rearranged to form 6 to 12 profiles of dense lamellae concentric to the endonuclear channel (Figures 5a, b, 6a, b).

By this time, the acrosome had turned 90° so that its major axis became parallel to the cell's polar axis (Figure 6a). In the middle piece, new mitochondrial annulae were added until a mitochondrial layer 6 to 8 μm deep was reached. The differentiated principal piece contained a granular material that was arranged into nine rosettes spatially related to the nine pairs of microtubules of the axoneme. These rosettes reacted positively to Thiery's test for polysaccharides (Figure 7).

Mature Spermatozoon

At the light microscopic level, the spermatozoa of *Chorus giganteus* appeared as simple filiform structures averaging 100 μm in length. The head, which had a densely stained, cylindric and pointed nucleus, corresponded to 50% of total sperm length (Figure 8). These proportions agree with measurements reported by Amín *et al.* (1984).

With SEM, the different segments of the spermatozoon could not be distinguished and the diameter appeared rather uniform along its length. In the apical region of the head, an acrosome was evident (Figures 9, 10).

In TEM observations, all mature spermatozoa of *Chorus giganteus* were morphologically identical. The head, about 50 μm in length, was 0.4 μm in diameter at the anterior end and 0.7 μm at the posterior end. At the top of the head, a cylindric-conic acrosome, about 1.2 μm in length, was observed. Between the outer and the inner acrosomal membrane a homogeneous granular material was present. Adjacent to the outer acrosomal membrane this material was condensed, forming crests of helicoidal appearance. The inner acrosomal membrane defined a conical subacrosomic space containing dense material, apparently a remnant of the proacrosomic granule (Figures 11, 12).

The nucleus, about 50 μm in length, was perforated by an endonuclear channel that ended blindly at its anterior end. The endonuclear channel was uniformly 0.2 μm in diameter. In mature spermatozoa, the chromatin, previously seen as concentric lamellae, appeared as a compact mass of high electron density. The axial filament, which originated from the centriole situated in the apex of the endonuclear channel, had the typical 9+2 configuration (Figures 12–14).

The middle piece, 6–8 μm in length and 0.7 μm in diameter, was characterized by a mitochondrial sheath tightly packed around the axoneme. The mitochondrial complex was surrounded by a common membrane produced by the breakdown and subsequent fusion of the outer mitochondrial membranes. At the distal end of the middle piece, the annulus, or Jensen's ring, was observed (Figures 14–16).

The principal piece, at about 42 μm in length, was the longest medial part of the flagellum and tapered from 0.5 μm in diameter to about 0.3 μm at the beginning of the end piece. The glycogen particles that surround the axoneme were present all along the principal piece, but decreased in number distally (Figures 15, 16).

The end piece, about 2 μm in length and 0.25 μm in diameter, was the short posterior part of the flagellum. It was formed by the axoneme and the surrounding plasma membrane (Figures 17, 18).

DISCUSSION

Our findings provide new evidence regarding some relationships among sperm morphology, the nature of the medium in which fertilization occurs, and the presence of nutritive eggs.

Spermiogenesis in *Chorus giganteus* shows characteristics similar to those described for *Nucella lapillus, Colus stimpsoni,* and *Concholepas concholepas* (Walker & MacGregor, 1968; Walker, 1970; West, 1978; Huaquín & Bustos-Obregón, 1981). In all of these species, including *Chorus giganteus,* the morphogenetic changes of the differentiating spermatids are, principally, aggregation and condensation of chromatin, acrosome formation, changes in mitochondrial distribution, formation of an endonuclear channel, and elongation of the axial

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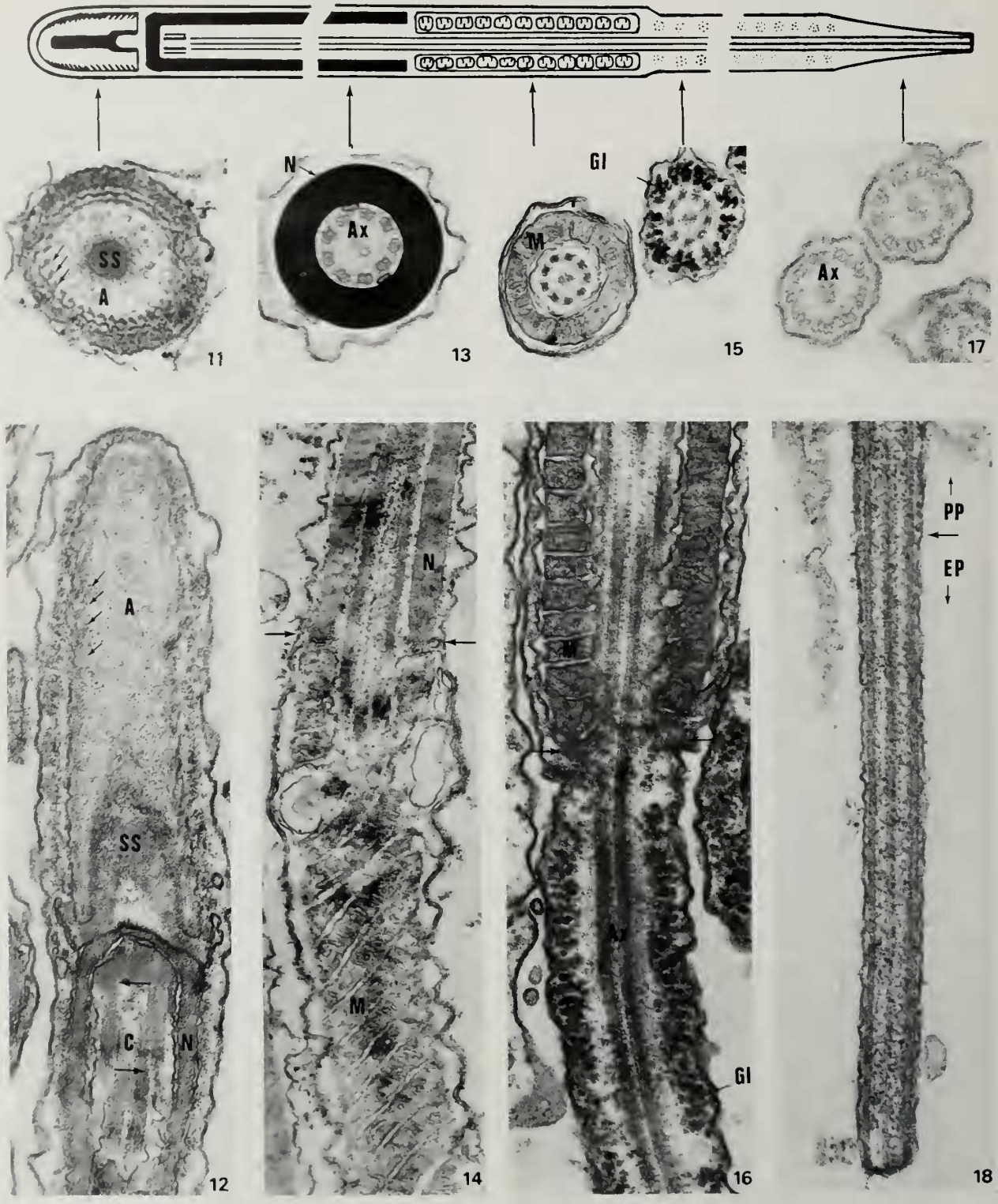
Explanation of Figures 7 to 10

Figure 7. Cross section of the principal piece of a late spermatid, stained by Thiery's method. Arrows indicate glycogen particles. ×110,000.

Figure 8. Smear of mature spermatozoa, observed with light microscope. ×800.

Figure 9. SEM micrograph of the whole sperm. H, head; MP, middle piece; T, tail. ×2200.

Figure 10. SEM micrograph of the head. A, acrosome; N, nucleus. ×28,000.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filament. However, some particular differences can be mentioned. In the early spermatid development of *Chorus giganteus,* chromatin is irregularly distributed throughout the nucleus, with a vacuolized zone around the nucleolus. In *Colus stimpsoni,* there is a similar distribution of chromatin, leaving a large zone of clear nucleoplasm near the nuclear envelope that contains a single dense "granule" (WEST, 1978). This vacuolized zone is called the polar nucleoplasm. The polar nucleoplasm moves to the anterior pole, forming a polar nucleoplasmic cone toward which the anterior end of the endonuclear channel projects. We suggest that the "dense large granule" described for *Colus stimpsoni* has a similar nucleolar morphology.

In *Chorus giganteus* the granules of chromatin fuse, eventually forming filaments and, then, lamellae concentrically arranged around the flagellum. At the end of spermiogenesis the lamellar arrangement is lost and the nucleus develops a tubular shape with a dense and homogeneous appearance. In *Nucella lapillus,* observations of the breakdown of the mature sperm's nucleus reflect the condensation pattern of chromatin described by WALKER (1970) as a "lamellar" type. This "lamellar" arrangement of the chromatin could be a common feature with *Chorus giganteus.*

In the apical end of the early acrosome of *Colus stimpsoni,* a ring of electron-dense material and a dense layer below the base are formed. During further maturation the ring disappears and the basal layer forms a subacrosomal plate between the acrosome and the nucleus. This plate is perforated by a single hole which is penetrated by a portion of the centriole (WEST, 1978). The electron-dense ring and the subacrosomal plate have not been seen in the maturing acrosome of *Chorus giganteus, Nucella lapillus,* or *Concholepas concholepas.*

Although there are differences in spermiogenesis, the morphology of the mature spermatozoa in *Chorus giganteus* is similar to that described in *Nucella lapillus* (WALKER & MACGREGOR, 1968; WALKER, 1970; RETZIUS, 1906), *Colus stimpsoni* (WEST, 1978), and *Concholepas concholepas* (HUAQUÍN & BUSTOS-OBREGÓN, 1981). All of these species exhibit the presence of type II (modified) spermatozoa, but not atypical sperm. Typical type II spermatozoa in the above-mentioned species having internal fertilization (AMÍN *et al.,* 1984; GUZMÁN *et al.,* 1972; WEST, 1979; GALLARDO, 1979; GALLARDO & PERRON, 1982) support the possibility that sperm morphology and mode of fertilization may be interrelated phenomena.

This correlation is supported by the presence and disposition of structures that may be involved in the transport and maintenance of spermatozoa within the female genital tract. In *Chorus giganteus,* the female genital system conforms to the general pattern found in other neogastropods, with a few exceptions (unpublished data). The bursa copulatrix receives sperm at copulation and passes them on to the seminal receptacle or directly transfers them along the ventral channel of the pallial oviduct. It appears that fertilization occurs in the albumin gland at the posterior end of the pallial oviduct (HOUSTON, 1976). At this level the eggs are surrounded by two special envelopes, the vitelline membrane and the chorion, and presumably additionally by a thin cover of albumin.

In the mature sperm of *Chorus giganteus,* the continuation of the flagellum into the endonuclear channel allows for undulating movements throughout the head length. This activity certainly depends on the numerous mitochondria present in the middle piece. LONGO & ANDERSON (1970) have postulated that glycogen particles that surround the axoneme in the main piece represent the storage of endogenous substrate to be used by the mitochondria for ATP formation. This provides an energy source either for sperm movement along the female genital tract or for sperm maintenance in the seminal receptacle.

The granular material of crested appearance that is adjacent to the inner surface of the outer acrosomic membrane could play a role during fertilization in *Chorus giganteus.* In lampreys, subacrosomal material forms a long helical fiber that is extruded to form the core of a true acrosomal tubule during the acrosome reaction (NICANDER & SJÖDEN, 1968). This acrosomal tubule makes the first contact with the egg surface, as in many invertebrates (COLWIN & COLWIN, 1967). The crest of helicoidal appearance in the acrosome of *Chorus giganteus*

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Explanation of Figures 11 to 18

Figure 11. Cross section of the acrosome (A). Arrows indicate dense crests of the acrosome; SS, subacrosomal space. ×103,000.

Figure 12. Longitudinal section of the acrosomal and nuclear regions. A, acrosome; C, centriole; N, nucleus; SS, subacrosomal space; arrows indicate dense crests of acrosome. ×62,000.

Figure 13. Cross section of the nucleus (N). Ax, axoneme. ×62,000.

Figure 14. Longitudinal section of the boundary region (arrows) between the nucleus (N) and middle piece. M, mitochondria. ×53,000.

Figure 15. Cross section of the middle piece and principal piece. Gl, glycogen particles; M, mitochondria. ×45,000.

Figure 16. Longitudinal section of the middle piece and principal piece. Ax, axoneme; Gl, glycogen particles; M, mitochondria; arrows show Jensen's ring in the boundary between the middle piece and the principal piece. ×56,000.

Figure 17. Cross section of the end piece. Ax, axoneme. ×96,000.

Figure 18. Longitudinal section of the limit (arrow) between principal piece (PP) and end piece (EP). ×50,000.

could represent a similar adaptation to promote the penetration of the spermatozoon through the egg investments.

The absence of sperm dimorphism and the presence of nutritive eggs in *Chorus giganteus* (GALLARDO, 1980), *Colus stimpsoni* (WEST, 1981), *Nucella lapillus* (WALKER, 1970), and *Natica catena* (ANKEL, 1930) disagree with the proposition that the atypical spermatozoa play a role in the determination of nutritive eggs. Alternative explanations could be those proposed by PORTMAN (1931a), according to whom there is a "dimorphism of physiological nature" based on differences in nuclear condensation during spermatogenesis in *Thais lapillus,* or by RAVEN (1970) who implicated follicle cells in influencing ooplasmic localization in *Lymnaea stagnalis.*

ACKNOWLEDGMENTS

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